Inhibition of micro-RNA 126 to Prevent Kasabach-Merritt Phenomenon in Endothelial Cell Tumors

Research Thesis

Presented in partial fulfillment of the requirements for graduation *with research distinction* in Biology in the Undergraduate Colleges of The Ohio State University

by

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April 2018

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Relevant Terminology:

EC	Endothelial cell
HE	Hemangioendothelioma
KMP	Kasabach-Merritt Phenomenon
miRNA	micro-Ribonucleic Acid
mTOR	mammalian Target of Rapamycin

Introduction:

Endothelial cell (EC) tumors, the most common soft tissue tumors in children, occur in 1-2% of all children worldwide and present more often in Caucasian child with a rate of up to 10% (Gordillo & Sen, 2011; Mulliken et al., 2004). Most EC tumors appear on the heads and necks of infants, where 50% leave residual deformities even after involution or treatment (Biswas et al. 2015). Although the vast majority of EC tumors are classified as benign, such as infantile hemangioma, there is still a wide spectrum of malignancy ranging from intermediate-grade hemangioendotheliomas to aggressive high-grade angiosarcomas (Gordillo & Sen, 2011; Wagner et al., 2017).

Hemangioendotheliomas (HE), as known as Kaposiform Hemangioendotheliomas (KHE), are primarily classified as an intermediate-grade malignancy because of its capacity to develop Kasabach-Merritt Phenomenon (KMP). Distinguishing HE tumors from hemangiomas are critical for helping prevent KMP, but are sometimes difficult because of their variety of clinical morphologies (Fernandez et al., 2009). However, HE tumors occur more frequently on the trunk and extremities, appear more purple, and typically do not resolve or disappear on their own compared to hemangiomas (Mulliken et al., 2004). Radiological imaging, magnetic resonance imaging, or ultrasound may be performed to help identify the types of EC tumor (Fernandez et al., 2009). Fortunately, some pharmacologic therapies, such as interferons or high dose steroids, can treat both hemangioma and HE. However, HE are typically treated more aggressively with chemotherapies, such as vincristine (López et al., 2009). HE tumor resection is a definitive treatment. However, HE tumors may cross tissue lines from dermis to subcutis, fascia, muscle, and bone making it almost impossible to resect because of the high probability of

severe blood loss (Croteau et al., 2013; Blatt et al., 2010). All of these treatments have high-risk side effects. Therefore, research needs to focus on understanding the causes of this infantile tumor to establish an effective and safe treatment.

I. Background on Kasabach-Merritt Phenomenon:

The first described case of KMP was in 1940 by Haig Kasabach and Katherine Merritt. KMP occurs when the HE tumor sequesters blood and platelets, as well as exhibits consumptive coagulopathy, which is a marked reduction of platelets with depletion of coagulation factors in peripheral blood (Fernandez et al., 2009; Gordillo & Sen, 2011). Without treatment, infants with KMP may experience anemia, heart failure, and excessive bleeding, which can all lead to death (Gordillo & Sen, 2011). KMP has a mortality rate of up to 30% (Fernandez et al., 2009). Fortunately, HE tumors occur without KMP in 29-43% of cases (Ryu et al., 2017). It should also be noted that disseminated intravascular coagulopathy (DIC), the activation of clotting cascades, is commonly termed as a differential diagnosis for KMP; however, DIC may occur in a variety of tumors or malformation, where as KMP forms only in HE tumors (Fernández et al., 2009). Upon complete blood count analysis, hemoglobin, hematocrit, and platelet levels should be low to determine the diagnosis of KMP, as well as other clinical features associated with consumptive coagulopathy.

The molecular mechanisms for KMP are not well defined, resulting in less knowledge about effective and safe treatments. Possible first-line treatments for HE tumors and KMP are corticosteroids, which have variable response rates and approximately one-third of patients do not respond to this treatment alone (Ryan et al., 2009; Blatt et. al 2010). A proposed third-line treatment is vincristine, which has shown to be a more effective and safer treatment. New

findings have discovered that low-dose radiotherapy may also help treat KMP in severe cases (Ryan et al., 2009; Yamamoto et al., 2016). Recent studies have proposed a new, more effective treatment with Sirolimus, as known as Rapamycin, for KMP (Blatt et al. 2010; Kai et al., 2014). However, the noted side effects include mucositis, peripheral edema, hypertension, hypertriglyceridemia, hypercholesterolemia, and pneumonitis in rare cases (Blatt et al. 2010). Rapamycin treatment is advised to be administered topically because systemic treatments can lead to greater side effects (Medici & Olsen, 2012). This treatment is still being tested for its effectiveness before it can be provided as a first-line therapy, but evidence has exhibited that it is safer than some treatments for KMP (Wang et al., 2015). Overall, rapamycin provides new knowledge into the molecular etiologies of KMP.

II. Molecular Mechanisms Involving Micro-RNA 126

HE tumors are described as an angiogenic disease in which abnormal increases in capillary vessels result in increased tumor size and trapped platelets to form KMP (Ezekowitz et al., 1992). A key angiogenic agent in EC is micro-RNA 126 (miR126), which maintains vascular development, regeneration, and integrity as well as promotes angiogenesis (Fish et al., 2008; Sinha et al., 2015). Micro-RNAs (miRNAs) are noncoding small RNA molecules (~20-25 nt) that have the ability to regulate expression of genes through targeting messenger RNAs (mRNA) post-transcriptionally (Fish et al., 2008; Suárez & Sessa, 2009). These molecules can be described as an upstream “hub” capable of targeting a variety of downstream sites (Biswas et al., 2017). miRNAs can bind to the three prime un-translated region (3’UTR) and also the 5’UTR region of mRNAs to either repress or activate (less common) translation or degrade the mRNA molecule (Wu and Belasco, 2007). miR126, in particular, has been found to target several

coding gene pathways, such as targeting insulin receptor substrate-2 in insulin-secreting β-cells and vascular endothelial growth factor-A (VEGF) in gastric cancer, by binding to the 3'UTR of the mRNA (Lui et al., 2014; Chen et al., 2014).

Studies showed that miR126 mutations in mice result in diminished angiogenesis and increased mortality following coronary ligation for myocardial infarction modeling (Wang et al., 2008). Therefore, miR126 is important to vessel formation and mice survivability. miR126 plays an important role in the upstream activation of VEGF pathway, which is responsible for further angiogenic signaling and vessel formation. By repressing negative regulators of the VEGF angiogenic pathway, such as Sprouty-related protein (SPRED1) and phosphoinositol-3 kinase regulatory subunit 2 (PIK3R2/p85-beta), miR126 is a vital aspect of this pathway throughout the body (Sinha et al., 2015). Therefore, by applying this concept to HE tumors, targeting miR126 processes upstream may provide a new framework for an effective and safe treatment.

III. Role of mammalian Target of Rapamycin (mTOR) and Phosphoinositide-3-kinase regulatory subunit 2 (p-85β) in HE tumors and KMP

As discussed previously, Sirolimus, also known as Rapamycin, is a proposed treatment for HE tumors because it targets mammalian Target of Rapamycin (mTOR), a protein that activates angiogenesis pathways like VEGF (Wang et al., 2016; Keppler-Noreuil et al., 2016; Blatt et al., 2010). Target of Rapamycin (TOR) was first discovered in yeast, *Saccharomyces cerevisiae*, when studying the fungicide rapamycin. Later mammalian studies discovered mTOR, which had similar biochemical properties to TOR in yeast and was inhibited by Rapamycin

(Pópulo, Lopes, & Soares, 2012). mTOR is a protein serine/threonine kinase that has been shown to facilitate EC proliferation (Wang et al., 2017). Structurally, mTOR is constructed for two functionally and structurally distinct complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (Watanabe, Wei, & Hunag, 2011). mTORC1 is the complex responsible for positively regulating mTOR kinase activity and rapamycin-sensitivity (Pópulo, Lopes, & Soares, 2012). Rapamycin, as an inhibitor of mTOR, decreases angiogenesis through decreasing angiogenic responses to VEGF, which inhibits the growth of HE tumors and therefore prevents KMP (Keppler-Noreuil et al., 2016; Wang et al., 2017). Hyper-activation of mTOR is found in 80% of human cancers and Rapamycin is an expected effective treatment (Watanabe et al., 2011).

The cellular processes of mTOR are found in conjuncture with phosphoinositide-3-kinase (PI3K) and protein kinase B (AKT) known as the PI3K/AKT/mTOR pathway as seen in **Figure 1** (Keppler-Noreuil et al., 2016). This pathway promotes normal cellular functions such as proliferation, growth, cell survival, angiogenesis, and metabolism. Studies have been trying to understand the molecular mechanisms behind this pathway to establish treatments for a variety of diseases. Many regulatory molecules for this pathway have been identified, such as phosphoinositide-3-kinase regulatory subunit 2 (p85β/PIK3R2) and tuberous sclerosis 1 (Tsc1) (Mirzaa et al., 2015; Agudo et al., 2014). PIK3R2 is the gene that encodes for p85β and *Tsc1* encodes for the protein Tsc1. miR126 has been shown to target both of these proteins by binding to the 3'UTR of their mRNA, which decreases protein expression (Sessa et al., 2012; Agudo et al., 2014). In this current project, p85β, as an inhibitor of mTOR, was analyzed by treatment with

miR126 inhibition. **Figure 2** demonstrates the proposed interaction between miR126, p85β, and mTOR.

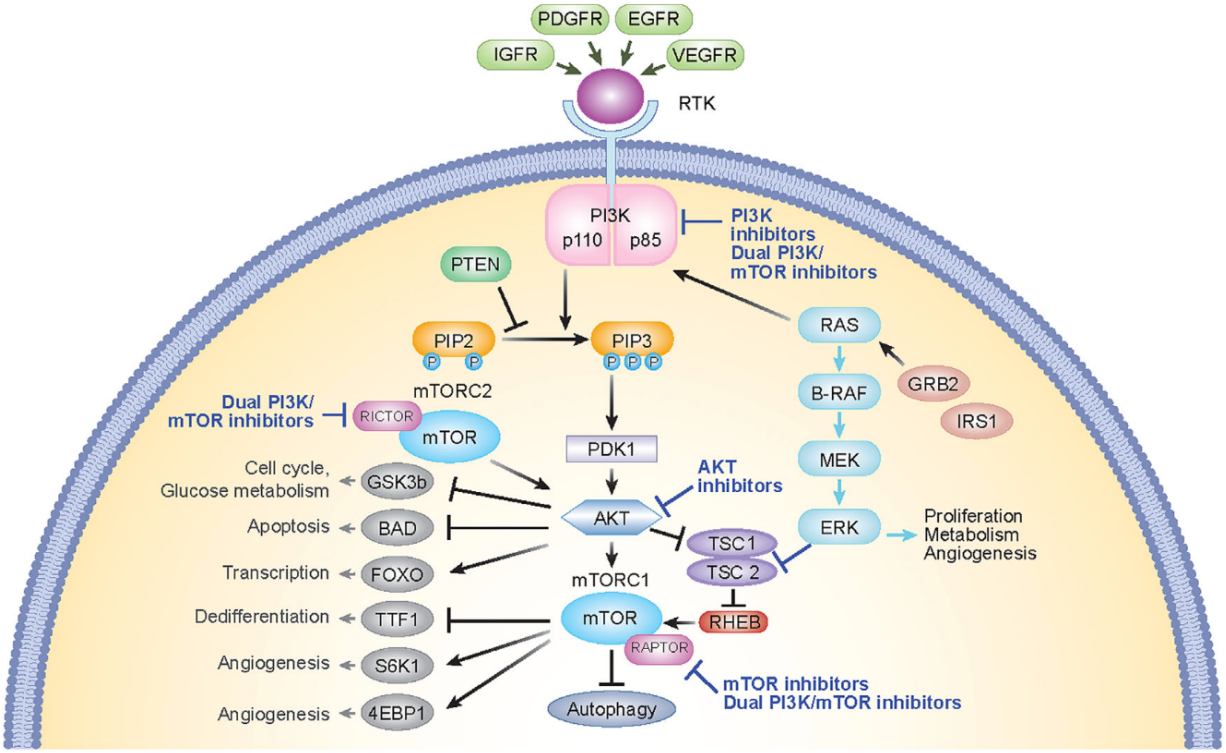

Figure 1. **PI3K/AKT/mTOR pathway interaction diagram.** Overview of the complex pathway that controls proliferation, growth, cell survival, angiogenesis, and metabolism in endothelial cells. Possible inhibitors are noted. *Diagram taken from: Keppler-Noreuil et al., 2016.*

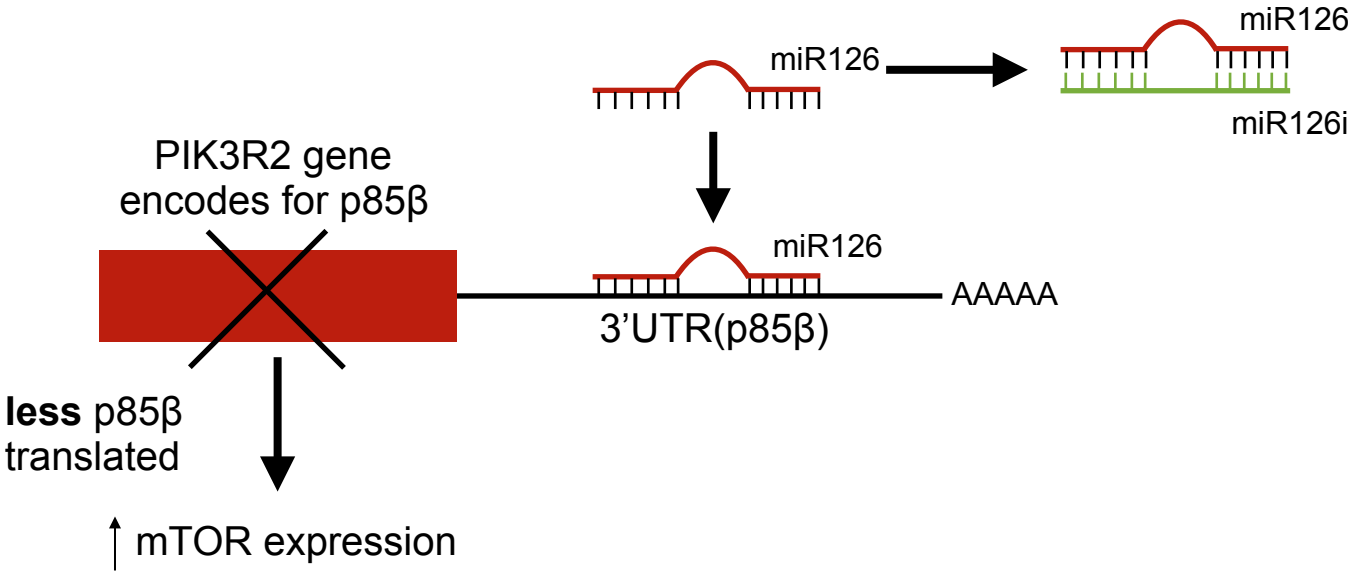

Figure 2. **Proposed mechanism of miR126 relationship with p-85β and mTOR.** Interaction diagram of miR126 with the 3 prime untranslated region (3'UTR) of phosphoinositide-3-kinase regulatory subunit 2 (p-85β) and the effects on mTOR. Inhibitor of miR126 = miR126i

IV. HE Tumor Model and Treatment

For analyzing HE tumor proliferation and KMP mechanisms *in vivo*, the first established and validated endothelial cell line was called hemangioendothelioma endothelial cells (EOMA) (Hoak et al., 1971). When injected subcutaneously into 129 P/3 mice, HE tumors arise spontaneously with 100% efficiency and effectively model human HE tumors (Gordillo et al., 2004). These tumors also model KMP found in humans and therefore is the given model for this project. After growing the tumor over 3 days, the mice are anesthetized and treated with topical tissue nano-transfection (TNT). Simply stated, topical TNT is similar to electroporation for gene transfer, however on a much smaller scale, where electric pulses allow the membranes of cells to open allowing for molecules to enter the cells (Fei et al., 2013; Gallego-Perez et al., 2017). In this project, TNT was used to administer anti-miR126 and anti-control products into the cell to study the effects of miR126 inhibition.

Aim:

The purpose of this project is to test whether miR126 inhibition prevents KMP through repression of mTOR.

Proposed Mechanism:

miR126 is an upstream stimulator of mTOR that promotes angiogenesis, which increases HE tumor size and susceptibility to KMP. Activation of mTOR is miR126 dependent because miR126 inhibits p-85β resulting in mTOR activation. Inhibition of miR126 results in decreased mTOR expression by de-repression of p-85β.

Methods:

Cell Culture:

Mouse hemangioendothelioma endothelial (EOMA) and non-tumor murine aortic endothelial cells (MAE) were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin and incubated at 37˚C and 5% CO_2 in T75 flask or seeded in 12-well plates for further treatment.

In Vitro Transfection of miRNA:

EOMA cells taken from T75 plates were seeded in 12-well plates (1 x 10^5 cells/well) in antibiotic free medium for 24 hours at same incubation standards as described above before treatment. DharmaFECT^{TM}1 transfection reagent was used to transfect control or miR126 inhibitor (Thermo Scientific Dharmacon RNA Technologies). After incubation at 37˚C and 5% CO_2 for 72 hours, cells were washed, lysed, and collect for RNA/protein analysis.

RNA Extraction and Quantitative Real Time PCR:

From cell culture, EOMA and MAE cells in T75 plates and EOMA treated in 12-well plates were washed with Phosphate Buffered Saline (PBS) and centrifuged into a pellet. RNA was purified using miRVana miRNA isolation kit according to manufacturing standards (Ambion/Invitrogen). After measuring the RNA concentration, miRNA were reversed transcribed to cDNA by using the miR TaqMan assays and TaqMan microRNA reverse transcription kit according to protocol (Applied Biosystems/Invitrogen). Abundance of miRNA was quantified by using real-time

polymerase chain reaction (PCR) with Universal PCR Master Mix (Applied Biosystems/ Invitrogen).

Western Blot:

For immunoblotting, EOMA and MAE cells were lysed, proteins isolated, and concentration measured using bicinchoninic acid (BCA) protein assay. 25-40 µg of protein/lane for each sample were separated using 4-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Gel proteins was transferred onto a nitrocellulose membrane and then probed with rabbit monoclonal anti-p-85β antibody (1:1000 dilution), anti-(phospho)-mTOR antibody (1:1000 dilution), and anti-GAPDH antibody (1:5000 dilution). After a series of washing steps with Tris Buffered Saline (TBS) and Tris Buffered Saline with Tween 20 (TBST), bands were visualized through horseradish peroxidase-conjugated anti-rabbit IgG raised in donkey and horseradish peroxidase-conjugated anti-mouse IgG raised in sheep (Amersham Biosciences) at 1:2,000 dilution and chemiluminescence assay (Amersham Biosciences). Using ImageJ software, individual band intensity was measured by pixel densitometry.

Matrigel® Angiogenesis Assay:

EOMA cells that were transfected with control inhibitor or miR126 inhibitor were trypsinized and resuspended in media at 6 x 10^4 cells/ml. In a four-well tissue-culture plate, 500µl of cell suspension was seeded on growth factor-reduced, phenol red-free Matrigel, according to manufacturer's instruction, and incubated at 37˚C and 5% CO_2 overnight. 0.5µl of Calcein AM was used to fluorescently stain cell cytoplasms and incubated 37˚C and 5% CO_2 for 15 to 60

minutes. Zeiss Axiovert 200M microscope was used to capture fluorescent images of tube formation. Quantification of tube formation was analyzed using AxioVision LE software version 3.1 for three images per well to measure total area within the tube under 50X magnification.

Target Reporter Luciferase Assay:

MAE cells are trypsinized and seeded in a 12-well plate (0.075×10^6 cells/well) in antibiotic free medium for 24 hours before transfection. Then cells were transfected with control mimic and miR126 mimic for 72 hours at 37˚C and 5% CO_2. Abundance of miR126 was measured from the samples using previously described miRNA quantification method. After incubation, cells are re-transfected with pGL3-p-85β-3-UTR firefly luciferase expression construct (500 ng/sample) together with *Renilla* luciferase pRL-cmv expression construct (10 ng/sample) using Lipofectamine^{TM}LTXPLUS^{TM}reagent (Invitrogen). Luciferase activity was determined using luciferase reporter assay system. The data are presented as ratio of firefly to *Renilla* luciferase activity.

In Vivo *Studies*:

EOMA cells from T75 flasks were pelleted down from cell culture and washed with PBS. Trypan Blue Stain (0.4%) was used to measure cell count. A stock solution of cells in PBS (5×10^6 cells/100µl) was made and syringes were filled with 100µl for injection. Female 129P/3 mice, housed in clean environments in compliance with Institutional Laboratory Animal Care and Use Committee guidelines of The Ohio State University, are fed standard chow and water *ad libitum*. Around 6 to 8 weeks of age, dorsal sides of mice are shaved and then further hair removal with

Nair. Following this, mice were injected subcutaneously with vehicle EOMA using the prepared syringes. After 3 days, mice are treated by tissue nano-transfection with either anti-miR126 and anti-control. On the second day after the initial treatment (day 5 after injection), mice are again treated by issue nano-transfection with either anti-miR126 and anti-control. Complete blood counts taken from OSU Comparative Pathology and Mouse Phenotyping Core for days 0, 5, and 10. Hemoglobin, hematocrit, and platelet levels for each day were plotted on a box and whisker plot to compare control and treatment. **Figure 3** provides a timeline of *in vivo* treatment.

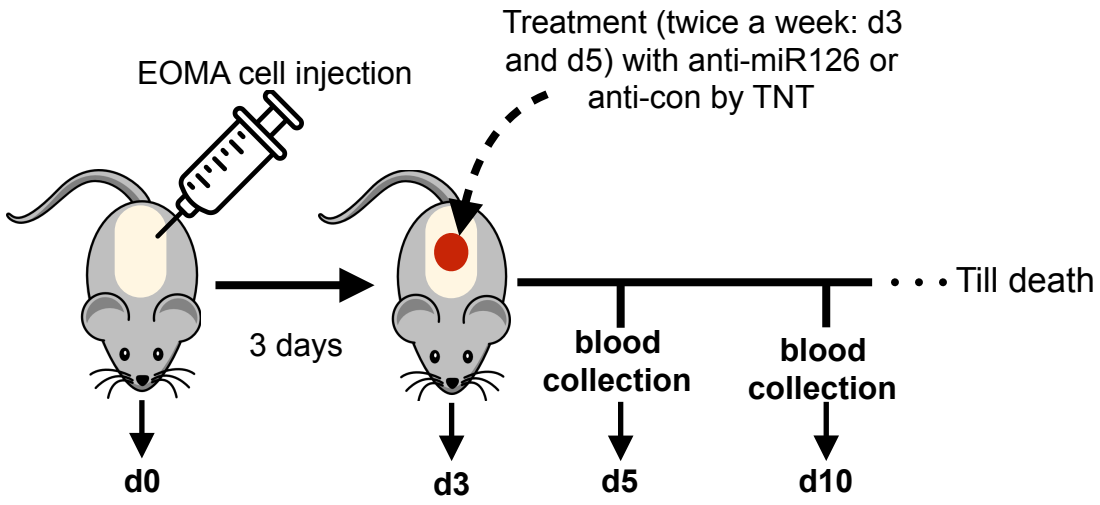

Figure 3. **Targeted Inhibition of miR126 in HE Tumors.** 129P/3 mice injected with EOMA cells. After 3 days are treated with anti-miR126 and anti-control through tissue nano-transfection (TNT). TNT is similar to electroporation for gene transfers. Complete blood counts were taken on day 0, 5, and 10.

Statistical Analysis:

All experiment were conducted at least twice. Two-sided two-sample *t*-tests were used to compare differences between two groups unless otherwise stated. All bar graphs represent means with error bars of one standard deviation. *p*-value of <0.05 is consider statistically significant.

Results:

To determine miR126 abundance in EOMA cells, real-time PCR was performed comparing EOMA and MAE cells. EOMA cells have a significantly higher abundance of miR126 compared to MAE cells (**Fig. 4-A**). After transfection with miR126 inhibitor and control inhibitor in EOMA cells, miR126 levels significantly decrease in the miR126 inhibitor group, as expected (**Fig. 4-B**). To examine functionality of miR126 inhibition, Matrigel® assay was performed to test tube formation. Following transfection with with miR126 inhibitor and control inhibitor in EOMA cells, there was a significant decrease EOMA cell tube forming capacity with treatment of miR126 inhibitor compared to control inhibitor (**Fig. 4-C** and **4-D**). Inhibition of miR126 results in decreased angiogenesis in EOMA cells. Therefore, miR126 angiogenic factors are necessary for HE growth.

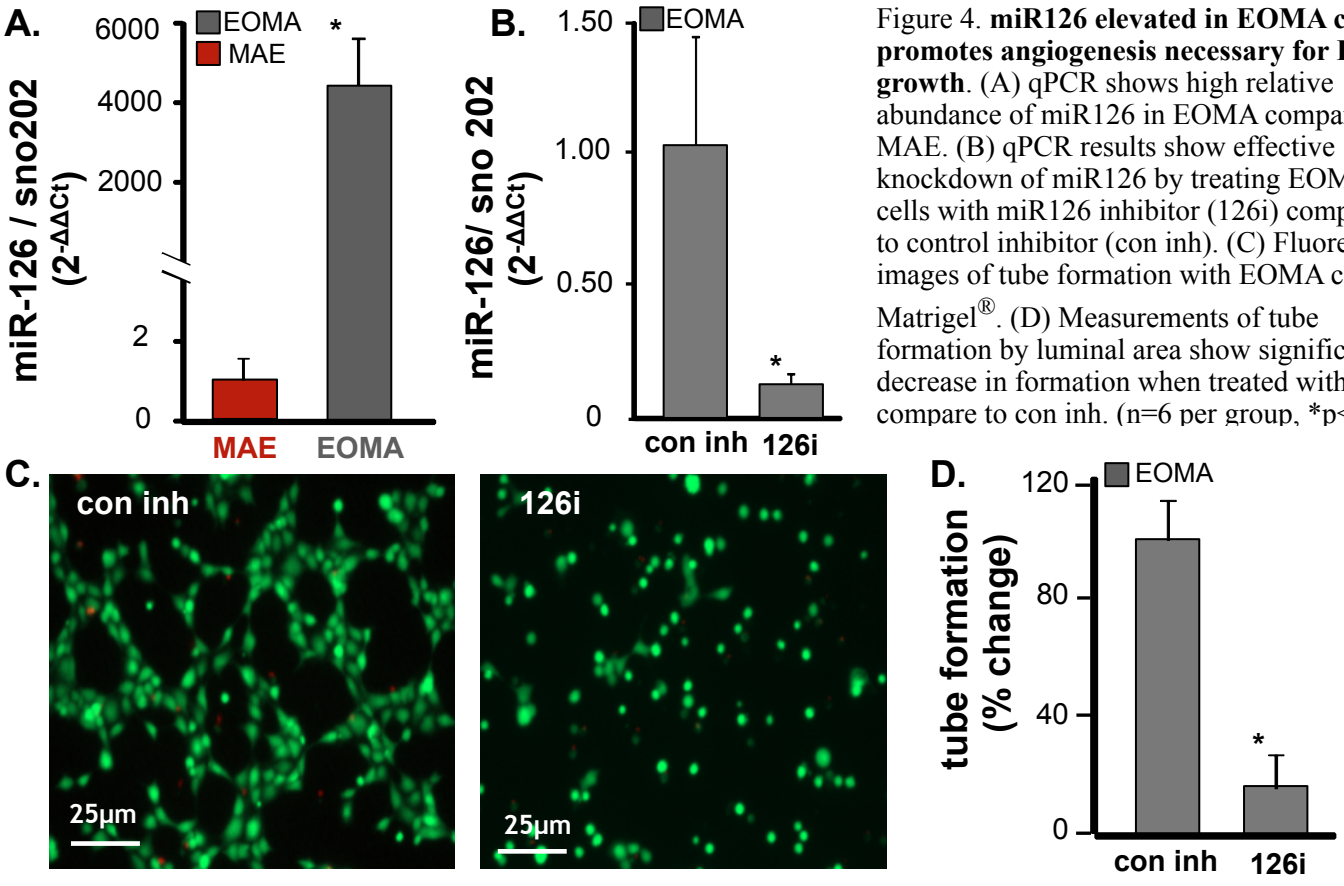

Figure 4. **miR126 elevated in EOMA cells promotes angiogenesis necessary for HE growth**. (A) qPCR shows high relative abundance of miR126 in EOMA compared to MAE. (B) qPCR results show effective knockdown of miR126 by treating EOMA cells with miR126 inhibitor (126i) compared to control inhibitor (con inh). (C) Fluorescent images of tube formation with EOMA cells on Matrigel®. (D) Measurements of tube formation by luminal area show significant decrease in formation when treated with 126i compare to con inh. (n=6 per group, *p<0.05)

Having identified increased abundance of miR126 in EOMA cells, the next step was to determine if miR126 targeted p-85β. This was tested by construction of a firefly luciferase reporter plasmid with the p-85β 3'UTR sequence. p-85β reporter plasmid was co-transfection with *Renilla* firefly luciferase reporter plasmids into MAE cells previously treated with miR126 mimic and control mimic. MAE cells were used instead of EOMA cells to control for the miR126 abundance. As expected, MAE cells treated with miR126 mimic had a higher abundance of miR126 compared to control mimc (**Fig. 5-A**). Following co-transfection, there was a significant decrease in luciferase levels in the MAE cells treated with miR126 mimic compared to control mimic (**Fig. 5-B**). This confirms that miR126 targets the 3'UTR of p-85β as diagramed in **Figure 2**.

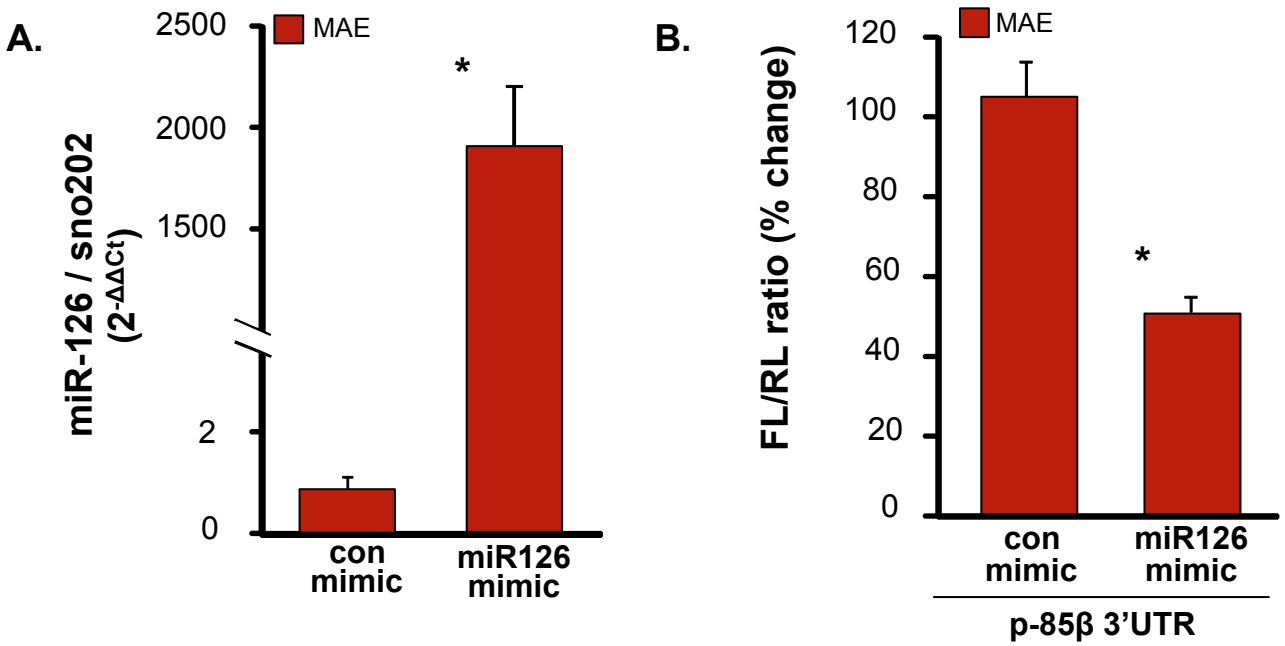

Figure 5. **miR126 targets 3'UTR of p-85β.** (A) Expression of miR126 in MAE shows expected results of higher miR126 levels with treatment of miR126 mimic compared to control mimic. (B) miR target luciferase reporter assay in MAE cells transfected with miR126 mimic or control mimic and then p-85β 3'UTR plasmid shows miR126 targets p-85β 3'UTR.(n=6, *$p<0.05$)

After determining that miR126 directly inhibits p-85β, identifying that p-85β and mTOR were miR126 dependent was the final *in vitro* procedure to verify. Expression of p-85β and mTOR were first analyzed in both EOMA and MAE cells to understand the inverse relationship between mTOR and p-85β. Immunoblot in **Figure 6-A** shows significantly lower expression of p-85β in EOMA cells compared to MAE cells. Phosphorylated mTOR (p-mTOR), the active form of mTOR, was blotted to show significant increase in mTOR expression in EOMA cells compared to MAE cells (**Fig. 6-B**). EOMA cells were then transfected with miR126 inhibitor and control inhibitor to examine the effects on p-85β and mTOR expression. **Figure 6-C** and **6-D** confirmed that with inhibition of miR126, expression of p-85β increases significantly and mTOR decreases significantly. This data supports the proposed mechanism of this work that p-85β and mTOR are miR126 dependent. As miR126 levels decreases, mTOR expression decrease because p-85β is de-repressed and targets mTOR.

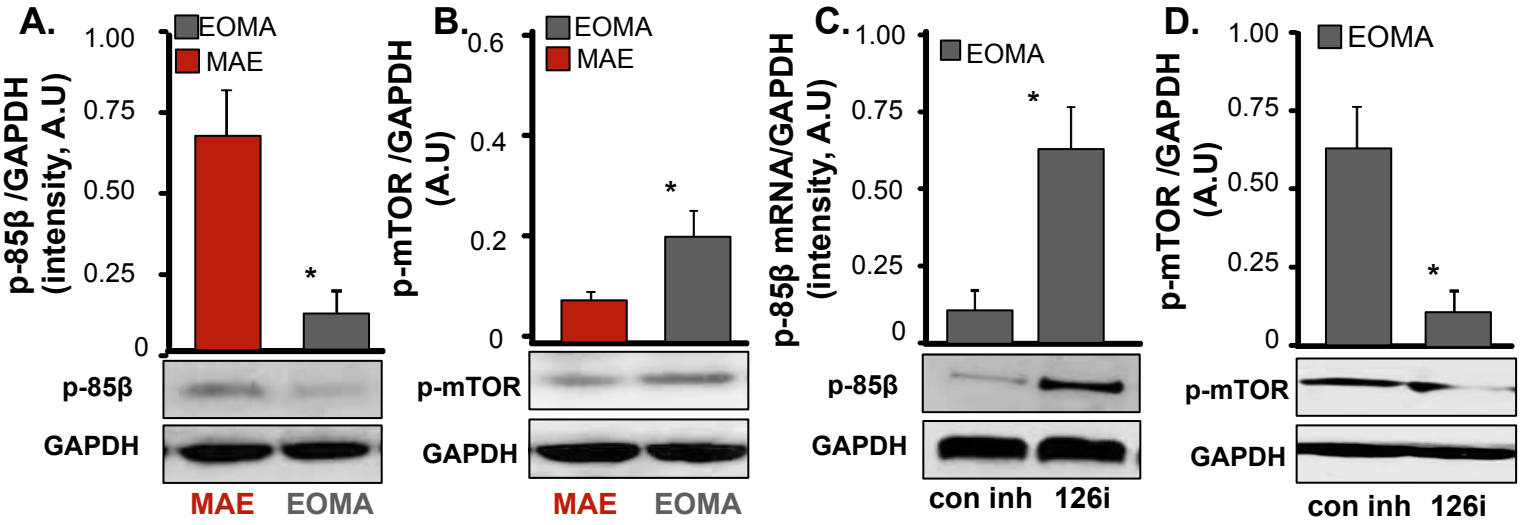

Figure 6. **p-85β and mTOR expression in EOMA cells are miR126 dependent.** Immunoblots of p-85β and (phospho) mTOR in EOMA, MAE, and EOMA treated with 126i and con inh. (A) p-85β has decreased expression in EOMA compared to MAE. (B) p-mTOR has increased expression in EOMA compared to MAE. (C) p-85β expression increases with 126i compared to con inh. (D) p-mTOR expression decreases with 126i compared to con inh. (n=3 per group, *p<0.05)

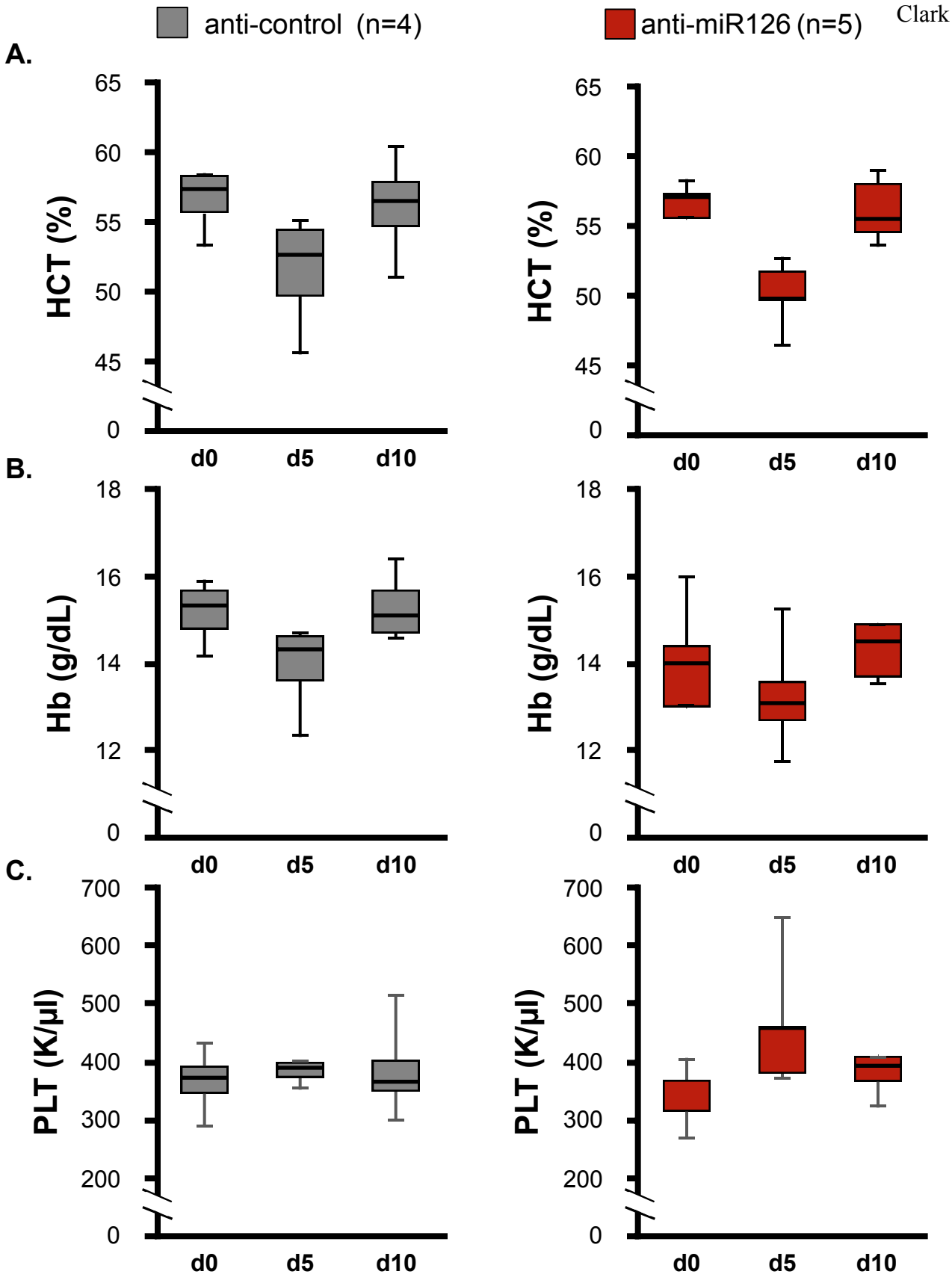

Figure 7. **Prevention of KMP.** (A) Hematocrit, (B) hemoglobin, and (C) platelet levels from day 0, 5, and 10 shows a slight trend of decreasing levels in anti-control group compared to anti-miR126 group. No significance can be drawn. This preliminary data indicates that treatment with anti-miR126 may prevent KMP.

The last experiment was to analyze miR126 inhibition *in vivo*, since it was found previously that inhibition of mTOR results in decrease HE growth and susceptibility to KMP in humans. 129P/3 mice were inject subcutaneously with EOMA cells and treated with tissue nano-transfection with anti-miR126 (n=5) or anti-control (n=4) on days 3 and 5. Complete blood counts was taken on days 0, 5, and 10 for hematocrit, hemoglobin, and platelets. In addition, blood smears were performed to identify reticulocytes. Box plots for hematocrit (**Fig. 7-A**), hemoglobin (**Fig. 7-B**), and platelets (**Fig. 7-C**) were created to show the medians, ranges, upper and lower quartiles, and positive and negative standard deviations. No statistical significance can be drawn from this preliminary data. There is a slight decreasing trend in the platelets of the anti-control treated group compared to the anti-miR126. Reticulocytes were seen in the blood smear of one anti-control treated animal (control 4) and not in anti-miR126 treated mice. This indicates possible hemolytic anemia and/or fragmented cells due to disseminated intravascular coagulation within this mouse, which is consistent with consumptive coagulopathy in KMP.

Discussion:

KMP in HE tumors is associated with significant morbidity and a mortality rate of up to 30% (Ryan et al., 2009). Fortunately, the incidence of KMP is low, where only < 1% of all children with vascular tumors are affected and 29-43% of HE tumor cases occur without KMP (Ryu et al., 2017). HE tumors with KMP utilize different cellular mechanisms to promote tumor growth through increased angiogenesis by activation of PI3K/AKT/mTOR and VEGF pathways (Keppler-Noreuil et al., 2016). Rapamycin, an inhibitor of mTOR, has been found to be an effective treatment for KMP and provided insight into its development. As the molecular etiologies for KMP are still not well studied, this project focused on deciphering some of its mechanisms.

Recently, a variety of miRNAs have revealed as biomarkers for many different molecular mechanisms and provided evidence as potential therapeutic targets for tumor management (Gordillo et al., 2014). To study the effects of miRNAs, EOMA cells, a validated model for HE tumors, were used. miR126, which has been found in abundance in several other tumor cells, was also found to have a significantly high abundance in EOMA cells compared to MAE cells (**Fig. 4-A**) (Ebrahimi et al., 2013). After miR126 inhibition, EOMA cells decreased in its ability to form tubes for angiogenesis (**Fig. 4-C and 4-D**). Vessel formation is key aspect for HE tumor growth and susceptibility to KMP. Therefore, regulating the vasculature process by inhibiting miR126 may provide useful for treating HE tumors.

For further examination on the role of miR126 in regulating angiogenesis, 3'UTR luciferase assays were performed on MAE cells with control and miR126 mimic to test miR126 binding to the mRNA of p-85β. In previous studies, miR126 has been shown to inhibit EC

expression of p-85β, a known negative regulator of mTOR (Sessa et al., 2012). In this project, miR126 was shown to target the 3'UTR of p-85β and significantly decrease its expression level (**Fig. 5-B**). This finding provides evidence that miR126 is important for mTOR activation, since miR126 inhibits p-85β, a mTOR repressor.

Immunoblots showed that p-85β expression is significantly lower in EOMA cells compared to MAE cells and mTOR expression is significantly higher (**Fig. 6-A** and **6-B**, respectively). Following treatment with miR126 inhibitor and control inhibitor in EOMA cells, p-85β expression levels significantly increased and mTOR expression levels significantly decrease with miR126 inhibition compared to control inhibitor (**Fig. 6-C** and **6-D**, respectively). mTOR and p-85β expression are miR126 dependent. As p-85β decreases mTOR expression, vessel formation will decrease through decreasing angiogenic responses to VEGF, which inhibits the growth of HE tumors and therefore prevents KMP.

Effects of miR126 inhibition was tested *in vivo*. As discussed previously, EOMA cells injected subcutaneously into 129P/3 mice spontaneously form HE tumors and all develop KMP without treatment. Following tumor formation, topical TNT with anti-miR126 and anti-control was used to study impact of miR126 inhibition. Complete blood counts (CBCs) taken throughout experiment (described in **Figure 3**) and plotted on box plots to compare the levels of hematocrit, hemoglobin, and platelet. KMP is characterized by low hematocrit, hemoglobin, and platelet levels as well as reticulocytes on blood smear analysis. Data does not show significance in decreased of hematocrit, hemoglobin, and platelet levels in the anti-control compared to anti-miR126 (**Fig. 7-A through 7-C**). There is a slight trend in decreasing platelet levels in anti-

control verse anti-miR126 groups. On one anti-control blood smear, there was identification of reticulocytes, which was not seen in anti-miR126 group.

There are no significant findings that provide evidence that KMP was prevented by inhibition of miR126. Several components may have affected the intended results. The experiment should have studied the mice until death with more time points for CBC and a survival analysis using a Kaplan-Meier curve could have been performed. To generate a greater statistical significant power, a larger sample size could have been used, which would have narrowed the range for standard error. This was also the first experiment to administer anti-miR126 into HE tumors through topical TNT. We are currently working on more *in vivo* experiments to resolve these challenges.

Future Directions:

MicroRNAs have been gaining more recognition for their molecular mechanisms, effects on vital cellular pathways, and applications as therapeutic targets. Two approaches to miRNA-based therapeutics have been tested. The first is developing miRNA silencers that are able to inhibit the production of miRNAs (Voglova, Bezakova, & Herichova, 2016). This has been shown to be effective in clinical trials for blocking liver-expressed miR122 in hepatitis C infections. The second approach is centered around miRNA replacement therapy, or miRNA mimics, to restore lost physiological function or lower abundance of miRNAs. This approach is still in clinical cancer trials focused around up-regulating miRNAs that function as tumor suppressors (Voglova, Bezakova, & Herichova, 2016).

After further murine studies that will hopefully discover miR126 importance in HE tumors and KMP, this miRNA could be used as a therapeutic target for treatment. In particular, as Voglova et al. described, development of a miR126 silencer that could be administered intravenously, orally, or topically. However, this method may have other serious side effects because miR126 is important in many pathway throughout the body, other than inhibiting p-85β to activate mTOR upstream.

Another method for targeting miR126 is the use of TNT in a clinical setting. Gallego-Perez et al. proposes that topical TNT may be administered all at one-time with a short treatment period, lasting only seconds. The anti-miR126 could be administered once into human HE tumors for effective, safe, directed treatment. This treatment could also be used in conjunction with current HE tumor treatments, such as corticosteriods, rapamycin, or vincristine (Ryan et al., 2009; Blatt et. al 2010).

Following this project, the most immediate direction to take is to determine the significant role of miR126 inhibition *in vivo*. As the first project to identify that mTOR and p-85β expression are dependent on miR126 abundance in HE tumors, there will need to be more studies to further analyze this relationship and related relationships. This project has provided more insight into the etiologies and treatments for KMP. With this new knowledge, this project has taken steps towards discovering a more effective and safer treatment for KMP to overall decrease the incidence of death due to this phenomenon.

Conclusion:

HE tumors are a type of EC tumors that have intermediate-grade malignancy because of their susceptibility to develop KMP, characterized by consumptive coagulopathy. Although KMP is rare, the mortality rate is up to 30% in infants that are diagnosed and most of these tumors do not resolve on their own, which presents a serious threat to the infant's well-being. This project was the first to identify that mTOR and p-85β expression are dependent on miR126 abundance in HE tumors that develop KMP. Inhibition of miR126 results in decreased mTOR expression by de-repression of p-85β, a negative regulator of mTOR. Further murine studies are being performed to establish how inhibition of miR126 may prevent KMP. After significantly identifying this relationship, miR126 may be used as a therapeutic target for HE tumor treatment with the goal of preventing further infant deaths from KMP.

Acknowledgements:

I would like to thank The Ohio State University Wexner Medical Center Department of Plastic Surgery and Surgery, The Ohio State University College of Arts and Sciences, The Ohio State University Davis Heart and Lung Institute. I would like to thank Dr. Gayle Gordillo allowing me to participate in her research lab for the past four years, introducing me to all her research ideas and findings, sparking my interest in the field of research, and supporting my ideas and concerns throughout my whole experience. Also I would like to thank Dr. Ayan Biswas for his commitment and constant support throughout my whole academic and research career. His guidance and knowledge of experimental protocols and molecular mechanism shaped much of these findings in this project.

I would like to thank Dr. Gregory Booton for his guidance throughout my academic career, as well as his support for advising this project. I would like to thank Ed Quinn for helping me through the proposal and research distinction process. I would like to thank The Ohio State University Undergraduate Research Office for providing information about research and providing opportunities to practice presenting my findings. Lastly, I would like to thank the National Institute of Health for the procedural and financial support of this thesis.

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